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ORIGINAL ARTICLE

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Courting virtual rivals increase ejaculate size in sticklebacks (*Gasterosteus aculeatus*)

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Abstract Sperm competition occurs when sperm of two or more males compete to fertilize a given set of eggs. Game-theory models of sperm competition predict that males, which face an increased risk of sperm competition, will invest more sperm in a mating. In the pair-spawning three-spined stickleback (*Gasterosteus aculeatus*), males attempt to steal fertilizations (i.e. to sneak) when they are in the courting phase, but not when they are parental. The behaviour of neighbouring males may therefore indicate the risk of sperm competition. We visually confronted males before spawning with computer animations of the same virtual stickleback showing two different behaviours: either courting (high risk of sperm competition) or brood-caring (low risk of sperm competition). We show that males invest significantly more sperm (absolutely and relatively with respect to available sperm in the testes) after the courting stimulus. The relative investment ratio between the courting and the brood-caring treatment was on average 1.75. Three-spined sticklebacks thus seem to assess the risk of sperm competition by the behaviour of neighbours and to adjust their ejaculate accordingly. This result suggests that the evolutionary force of sperm competition has led to precise mechanisms of future risk assessment.

Keywords Computer animations · Courtship · Ejaculate size · Sperm competition · Stickleback

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Introduction

Sperm competition (Parker 1970) can be seen as post-mating intra-sexual competition and is therefore regarded as an important force in sexual selection. A wide range of observational studies has shown that between and within species the ejaculate expenditure increases with the intensity of sperm competition (Baker and Bellis 1989; Bellis and Baker 1990; Gage 1994; Hunter et al. 2000; Nicholls et al. 2001; Shapiro et al. 1994; Stockley et al. 1997; Warner et al. 1995). This suggests that numerical superiority is an adaptive strategy in sperm competition, especially in species with external fertilisation, where the possibilities for cryptic female choice may be limited.

Since sperm are costly to produce (Dewsbury 1982), males are expected to allocate sperm carefully. Theory predicts that males should increase ejaculate expenditure when there is a "risk of sperm competition" (Parker et al. 1996, 1997). This term is defined for species in which females usually mate with a single male and sperm competition only occurs in a proportion of the matings (Parker et al. 1996). The amount of information a male has about sperm competition and the way this information is gained varies greatly among species. Information about past risk may be very precise (Wedell and Cook 1999), whereas in the case of sperm competition, which represents a future risk (i.e. externally fertilising fish), males have to assess the risk relying on more indirect cues in their environment, meaning that their assessment is more

error-prone. To manipulate the risk of sperm competition in the latter case, one has to anticipate how males assess the risk situation. Recently, several studies manipulated sperm-competition risk to test the theoretical predictions. Often, the presence or absence of potential competitors (Gage 1991; Gage and Barnard 1996; Fuller 1998) was the mechanism by which the risk of sperm competition was manipulated. A different approach is to manipulate age or experience of females (Simmons et al. 1993). In this study, we used a computer-animated virtual stickleback programmed to behave differently to manipulate the risk of sperm competition. Computer animations offer the possibility to solve several problems one often faces when manipulating risk of sperm competition. The rigorously repeatable set-up is non-interacting, thus excluding confounding effects like female choice and the operational sex-ratio between treatments, and the (absolute) quality of the stimulus is held equal.

Sperm competition in externally fertilising fish is a common phenomenon (Stockley et al. 1996; Taborsky 1998). Probably due to methodological constraints, experimental studies of sperm-competition risk or intensity are still rare. Ejaculate in the rainbow darter (*Etheostoma caerulum*) has been shown to be larger when other males (one or four) were present than when no male or female darter was around (Fuller 1998). Recent results align better with the theoretical prediction. Pilastro et al. (2002) showed, for two gobiid species, an increase in ejaculated sperm if one competitor was present, but a subsequent decrease with growing number of other males present. Similarly, in the European bitterling (*Rhodeus seiceus*), dominant males invested most (measured as the area of the clouds of semen) in preoviposition ejaculations if only one competitor was present (Candolin and Reynolds 2002).

Sperm competition in pair-spawning fish is mainly induced by sneaker males (Stockley et al. 1996; Taborsky 1998). Since not every neighbouring male is a potential sneaker in a given situation, males would benefit from the ability to assess the risk of sperm competition more precisely than merely by the number of neighbours. In this study, we examine whether male three-spined sticklebacks (*Gasterosteus aculeatus*) adjust their ejaculate size in response to the behaviour of a neighbouring male, using computer animations.

Male sticklebacks have a given amount of sperm available for the whole breeding season (Borg 1982). Spermatogenesis is inhibited during the breeding season, probably by increased levels of androgens (Borg 1982). Multiple-mated males have a smaller sperm store in their testes than virgin ones (Zbinden et al. 2001). Thus, sperm may be limited in this species and we would expect males to invest it strategically.

During the breeding season, males establish a territory in which they build a tunnel-shaped nest of plant material (Wootton 1984). After male courtship, females lay a clutch of eggs (40–295 per clutch; Wootton 1984) in the nest. Sneaker males attempt to steal fertilisations by spawning in foreign nests immediately after, or even before the nest owner. These sneakers are in most cases neighbouring territorial males without eggs in their own nests, whereas brood-caring males usually do not sneak (Goldschmidt et al. 1992; Jamieson and Colgan 1992; Mori 1995). Sticklebacks therefore experience a "risk of sperm competiton" situation. Between 15% and 25% of the nests contain sneaker-fertilised eggs (Jones et al. 1998; Largiadèr et al. 2001; Rico et al. 1992). The frequency of sneaking (and consequently of sperm competition) depends on habitat structure (Mori 1995; Sargent 1982), male density (Goldschmidt et al., 1992) and male quality (Jamieson and Colgan 1992; Mori 1995). Sneaking had regularly been observed, under laboratory and semi-natural conditions, in the population we used for this study.

Methods

Fish and experiment

Three-spined sticklebacks were collected during the spring 2000 migration on the island of Texel (Netherlands) and transported to the University of Bern (Switzerland). The fish were kept in a mixed-sex storage tank of about 320 l and supplied with running tap-water keeping the temperature between 16° and 19°C. Before the experiment, they were moved into a room with climate control and distributed between two 200-l tanks with running tap-water. Fish were kept at 16±1°C, under a light:dark regime of 16:8 h and fed to satiation with frozen chironomid larvae.

Males with developing breeding coloration were selected from the stock and placed separately in 15-l plastic aquaria that contained a Petri dish (ø 9 cm) with fine gravel and 8-cm-long pieces of green cotton twine, which served as nesting material. Males completed their nest between 1 and 3 days after isolation and were then tested within the next 2 days. To avoid any male–male interactions, aquaria were separated by opaque grey partitions.

We experimentally manipulated the risk of sperm competition by use of a computer animation of a courting stickleback male to simulate high risk, or a fanning male at the nest (i.e. brood-caring) to simulate low risk. After presentation of each simulation, test-males were allowed to mate. Sperm were then filtrated out of the nest and counted.

For the test, a male's aquarium with nest was placed alongside the computer screen which then showed the virtual scene used in the animations (Künzler and Bakker 1998) for half an hour. This virtual landscape showed a symmetric U-shaped simulation of the bottom of a pond, above which the sky was imitated. Following this period of acclimatisation, a ripe female in a 1-l container was placed in front of the male's aquarium for 5 min to stimulate sexual activity. After removal of the female, the male was shown one of the two computer animation sequences (described below). Then, the ripe female was allowed to spawn with the test male. To avoid disturbance of the fish by the observer, a black curtain separated the experimental set-up. Because the set-up was well illuminated and the ambient light was low, it was still possible to observe the fish through the curtain material.

Computer-animation sequences

The virtual fish was taken from the experiments described by Mazzi et al. (2003), and was a slightly modified version of the one previously described in detail by Künzler and Bakker (1998). A simplified version of the virtual stickleback can be seen with the freeware Quicktime Player on http://www.unifr.ch/biol/ecology/ebert/group/zbinden/movie.html. The courting sequence was taken from the experiments described by Mazzi et al. (2003), and showed

an average-size (52 mm) male with average coloration that was approaching a nest site, fanning and courting (zig-zag dance) in the direction of the focus male. For the fanning sequence, the same virtual male approached the nest site and then fanned the eggs repeatedly, with intermittent short head-ups to simulate normal vigilance behaviour. Both sequences lasted 155 s. Males reacted aggressively to both types of animated sequence by bites and bumps against the side of the tank where the virtual fish was shown. However, the level of aggressiveness was not quantified in this study. The occurrence of aggressive behaviour typical of male–male interactions (Rowland 1984) underlines the accuracy of the virtual stimuli. Computer animations have been shown before to work well in behavioural studies of sticklebacks (Bakker et al. 1999; Künzler and Bakker 1998, 2001; Mazzi et al. 2003). The suppositions for a correct usage of computer animation concerning colour perception and picture resolution are fulfilled in sticklebacks because their neurophysical eye-parameters are similar to those of humans, for which computer screens are built (reviewed in Künzler and Bakker 2001). Briefly summarised, the virtual stickleback has been produced by casting a male stickleback in resin, cutting this resin into thin slices (1 mm) and scanning these slices into a computer. Then the stickleback's body was virtually rebuilt by putting the slices together again. Spines, eyes and fins were completely computer-generated. The "ifish" was coloured realistically with respect to the body texture, the eye, and the throat colour. The "ifish's" movement was based on a video-recorded courting sequence of a stickleback male. Two video cameras simultaneously recorded the courting sequence from above and from the front of the aquarium. It was thus possible to determine x, y and z co-ordinates of every point of interest in the fish's behaviour, at any given moment. With this information, the virtual courting sequence was generated. Details on how the animation was manufactured, and technical details of the computer animations that were used, are described by Künzler and Bakker (1998).

Assessment of ejaculated and stored sperm

Immediately after the male had entered the nest to fertilise the eggs, we capped and removed the Petri dish containing the nest with eggs, sperm and about 35 ml of water. After using a filtration and staining procedure, described in detail in Zbinden et al. (2001), stained sperm were isolated on a dried Millipore filter ready for counting. Four segments of the dried filter, each about 1/8th of the filter's area, were mounted on a slide and cleared with immersion oil (Leong 1989). The areas of the filter were video-recorded and the sperm were counted after the experiment. The video sequences of the filter samples were randomised and prepared by a naive assistant, so we were blind with respect to treatment and a male's identity when sperm was counted. The number of sperm within a field (0.002 mm^2) was counted for 25 fields of each segment, using a Sony Trinitron screen linked to a light microscope (magnification: 1,000×). For practical reasons, five fields yielded one datapoint, resulting in 20 measurements per filter. Table 1 shows means and standard deviations of these data. Their distribution indicates that sperm are evenly distributed over the filter. Repeatability analyses of square-root-transformed data, performed for each treatment separately, reveals intraclass correlation coefficients (r_I) close to 1 (fanning treatment: $F_{16,323}$=298, r_I=0.94, P<0.001; courting treatment: $F_{16,323}$=775, r_I=0.97, P<0.001). This shows that our sperm counts are highly repeatable and give a good basis for calculating the number of ejaculated sperm. The detailed methods of counting and calculating sperm numbers are described elsewhere (Zbinden et al. 2001).

When the male had rebuilt its nest (between 1 and 3 days after the first trial), the test was repeated with the second test-sequence. The succession of the two test-sequences was randomised across males by tossing a coin for every odd replicate. The following pair replicate was treated in the inverse succession.

After the second trial, the sperm store in the male's testes was assessed with a Neubauer haemocytometer chamber, following the protocol given in detail in Zbinden et al. (2001). Testes were homogenised and sperm were counted in a Neubauer haemocytometer chamber. The amount of sperm in the testes (sperm store) before the trials was calculated by adding the ejaculate sizes to the number of sperm in the testes after the experiment.

Table 1 Mean and standard deviations of the number of sperm in the 20 measures per individual (17) and treatment. Estimations of the number of ejaculated sperm were based on these means

Individual	Courting		Fanning	
	Mean	SD	Mean	SD
1	155.30	15.13	55.35	15.18
2	426.95	19.00	122.60	9.25
3	71.75	14.71	38.60	5.78
4	108.80	8.97	120.60	17.84
5	115.10	13.28	103.00	9.73
6	79.65	11.40	42.25	5.78
7	81.05	14.11	44.95	12.28
8	155.10	15.59	65.25	9.24
9	33.20	8.04	8.15	2.91
10	136.35	13.50	91.05	13.01
11	218.05	24.25	81.00	19.02
12	56.25	8.69	33.45	6.34
13	95.40	7.43	64.35	11.43
14	119.55	11.72	98.80	10.02
15	232.30	18.75	203.50	27.73
16	11.75	3.26	31.85	5.58
17	73.45	12.71	114.75	15.53

Statistical analyses

Analyses were performed using the JMP IN 3.2.1 (SAS Institute) statistical package. Data were graphically checked to determine whether they conformed to the assumptions for parametric statistics, and transformations were applied if needed. Where no suitable transformation was applicable, non-parametric statistics were used. Paired t-tests were performed if the differences between the pairs of data were not different from normal distribution. Given P -values are two-tailed throughout.

Results

Males ejaculated significantly more sperm after having seen the courting male ($18.54\times10^6\pm3.41\times10^6$ sperm) than when the brood-caring male had been shown ($11.28\times10^6\pm1.67\times10^6$ sperm, means ± SE, Wilcoxon signed-rank test, n=17, T=60.5, P<0.01). The succession of the two stimuli, i.e. whether the courting or the fanning animation had been shown first, had no detectable effect on the difference between sperm investment of the courting test and the fanning test (Mann–Whitney U-test, n_{cf}=9, n_{fc}=8, Z=–0.63, P>0.5). However, as a consequence of the inhibited spermatogenesis, we expected less sperm in the testes before the second treatment than at the start of the experiment (Zbinden et al. 2001). We therefore calculated the ejaculate expenditure relative to the sperm store prior to the trial. The latter was estimated by the sum of the number of sperm in the testes after the experiment and either the second or both ejaculate sizes in the trials. This relative investment differed significantly between the two treatments (paired t-test: t=3.28, df=16, P<0.01; Fig. 1). One male considerably increased its investment

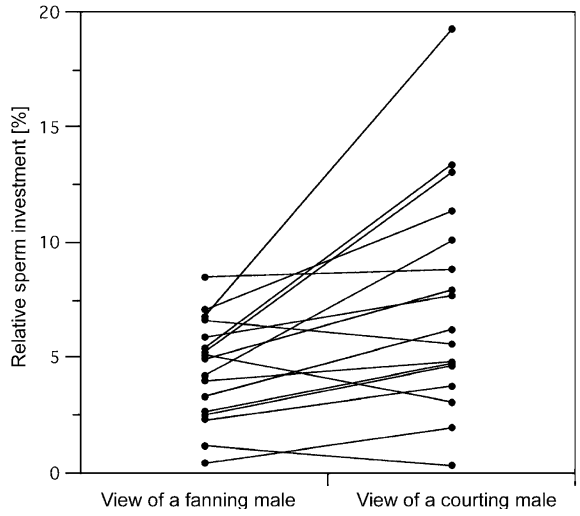

Fig. 1 Percentage of ejaculated spermatozoa, relative to the sperm store before ejaculation, in the two trials of each male three-spined stickleback (*Gasterosteus aculeatus*): prior to spawning, either a courting computer-animated male (to simulate high risk of sperm competition) or a fanning one (to simulate low risk of sperm competition) was shown to each male in random succession. Ejaculate size was estimated in the subsequent matings

by 12.5% of available sperm (Fig. 1). Exclusion of this extreme value does not affect the result (paired *t*-test: t=3.22, df=15, P<0.01). After being shown the fanning animation, males invested on average 4.6% (±2.2% SD) of their stored sperm. This is 3% (±3.7%) of their stored sperm less than males invested on average after having seen a courting animation (7.6%±4.8%). Consequently, the ratio of the relative investments between the courting and the fanning treatment equals on average 1.75 (±0.9 SD). So, compared to the fanning animation, males increased their investment in a mating by 75% when they had been shown the courting simulation. The median standard length of males was 57 mm (range 32–63 mm). Males larger than the median size reacted less strongly – though not significantly – to the risk of sperm competition, measured by the ratio of the ejaculate sizes in the two treatments (Mann–Whitney U-test, n_l=9, n_s=8, Z=1.78, P=0.075). The two females that mated with a male did not significantly differ in standard length, body mass or egg mass (paired *t*-tests, df=16, all P>0.5).

Discussion

This study provides experimental evidence that the behaviour of potential competitors influences the ejaculate size of male three-spined sticklebacks. They invest more sperm in a mating after having seen a courting male than after having seen a parental fish (Fig. 1). We interpret this finding as an adaptive reaction to an increased risk of sperm competition. Field studies have shown that stickleback males do not sneak after having entered the parental phase (Goldschmidt et al. 1992; Jamieson and Colgan 1992; Mori 1995). Consequently, we were able to manipulate the risk of sperm competition without changing the number of potential competitors. We show for the first time that risk assessment can be based on the behaviour of potential competitors, which may allow a finer tuning of sperm competition risk than just counting possible competitors. Our findings are concordant with recent theory about the risk of sperm competition in externally fertilizing species (Parker et al. 1996, 1997).

Simulation and manipulation of behaviour includes several traits. Any of these traits, or the effect of them all, can be the causal factor on which the strategic reaction is based. In our study the two stimuli differed in the fanning/courting time, general activity and mean distance to the focus fish. It is possible that one single component and not the behaviour as a whole was responsible for our result. However, differences in all these components are closely linked to the reproductive stage of the male. (Goldschmidt et al. 1992; Jamieson and Colgan 1992; Mori 1995). We do not know how sperm release is regulated in sticklebacks, nor the proximate causes of the strategic sperm investment. It is possible that courting rivals alter hormone levels, which influence sperm release.

Larger males reacted less strongly to the increased risk of sperm competition than smaller males, although this difference is not statistically significant. Courting male sticklebacks try to prevent sneaking by chasing away every male close to the nest before and after the mating. This often leads to vigorous attacks by the guardian male and even to fights between the guardian and a sneaking male. In such fights, larger fish may be better able to prevent sneaking in their nests than smaller males. Consequently, in our experiment, the risk of sperm competition may have been less high for larger fish, resulting in a tendency for a smaller increase in ejaculate size than found in the smaller fish. This suggestion is supported by the finding of Largiadèr et al. (2001) that larger stickleback males are less often the victims of sneaking than smaller males. Additionally, several studies on fish suggest a trade-off between ejaculate expenditure and aggression and/or mate guarding (Warner et al. 1995; Marconato and Shapiro 1996; Alonzo and Warner 2000)

To assess the size of an object, one has to be able to integrate its visual angle on the retina with an impression of its distance to the eye. In higher vertebrates, this assessment of depth in space is often done by stereoscopic vision. Due to the lateral position of the eyes, fish are generally thought to have a very small area of stereoscopic vision, if any at all. Despite that, they may be able to assess depth in space by other means (monitoring the accommodation system, motion parallax). Since we do not know how sticklebacks see three-dimensionally (or if they do at all), it is difficult to state how large the virtual fish was perceived to be. However, independently of the ability to perceive depth, it seems plausible that objects increasing in size (e.g. increasing visual angle) are perceived as approaching objects. Furthermore, our computer animation includes additional cues to give a

3-D impression, which may also be used by sticklebacks. First, size and shape of the virtual fish's shadow changes as it moves in the virtual pond. Second, approaching objects have more contrast and become clearer. We therefore believe that the movement of the courting male was perceived as such, and that the fanning stimulus simulated a brood-caring male back at its nest.

An individual's behaviour is an important source of information in interactions with others. Unfortunately, experimental work usually faces problems when trying to control or even manipulate behaviour. The use of computer animations as a tool in behavioural ecology opens up new possibilities in experimental studies, since manipulation of morphological and even behavioural traits becomes feasible where classical designs are constrained.

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